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Langerin Expressing Cells Promote Skin Immune Responses under Defined Conditions

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There are conflicting data in the literature regarding the role of epidermal Langerhans cells (LC) in promoting skin immune responses. On one hand, LC can be extremely potent APCs in vitro, and are thought to be involved in contact hypersensitivity (CHS). On the other hand, it seems counterintuitive that a cell type continually exposed to pathogens at the organism’s barrier surfaces should readily trigger potent T cell responses. Indeed, LC depletion in one model led to enhanced contact hypersensitivity, suggesting they play a negative regulatory role. However, apparently similar LC depletion models did not show enhanced CHS, and in one case showed reduced CHS. In this study we found that acute depletion of mouse LC reduced CHS, but the timing of toxin administration was critical: toxin administration 3 days before priming did not impair CHS, whereas toxin administration 1 day before priming did. We also show that LC elimination reduced the T cell response to epicutaneous immunization with OVA protein Ag. However, this reduction was only observed when OVA was applied on the flank skin, and not on the ear. Additionally, peptide immunization was not blocked by depletion, regardless of the site. Finally we show that conditions which eliminate epidermal LC but spare other Langerin+ DC do not impair the epicutaneous immunization response to OVA. Overall, our results reconcile previous conflicting data in the literature, and suggest that Langerin+ cells do promote T cell responses to skin Ags, but only under defined conditions. The Journal of Immunology, 2008, 180: 4722–4727.

Langerhans cells (LC) are a type of dendritic cells (DC) that constitute a dense network located in the epidermis (1). Due to this location, LC are thought to play a critical role in immune responses in the skin. Indeed, they have been shown to take up skin Ags and migrate to the skin draining lymph nodes (2) where they can potentially interact with T cells. Based primarily on in vitro studies, it is envisaged that LCs undergo a process of maturation coordinate with migration, where they up-regulate MHC, costimulatory, and adhesion molecules, and act as potent stimulators of naive CD4 and CD8 T cells when they arrive in the lymph node (3–5). Indeed, epicutaneous immunization strategies, which are presumed to involve LC, are effective at inducing functional CTL (6, 7) and have led to clinical trials with positive preliminary findings for influenza and travelers’ diarrhea (www.iomai.com) (8, 9).

Despite the unanimity of in vitro studies, the in vivo evidence supporting an immunomodulatory role for LC in immune responses that involve the skin is controversial. Recent studies of contact hypersensitivity using three distinct LC ablation models gave disparate results. Bennett et al. (10) and Kissenpfennig et al. (11) created gene targeted mice that expressed a diphtheria toxin receptor (DTR) under the control of the Langerin gene. When the toxin was given to receptor knock-in mice, LC were rapidly eliminated from the epidermis and s.c. lymph nodes. Contact hypersensitivity (CHS) was reduced after removal of LC in the first conditional ablation model, consistent with earlier work (12). However, CHS was unaffected by LC ablation in the second conditional model (11). Kaplan et al. (13) used a different strategy, where diphtheria toxin (DT) itself was expressed under the control of the human Langerin promoter in a bacterial artificial chromosome transgene. Such mice lack LC from birth, and showed an increased CHS response. Thus, the precise role of LC in the contact hypersensitive response remains unknown. In experiments using herpes virus and Leishmania infection of the skin, a Langerin-negative population of DCs, not the expected LC, was found to present Ag to T cells (14, 15). In studies of skin self-Ags, LC were shown to transport either model or endogenous self-Ags to the lymph nodes in the steady state (16). In data from our laboratory using a K14-OVAp model, this resulted in CD8 T cell-mediated autoimmune skin disease (17).

In this study, we used one of the conditional ablation models (11) and show that LC can play a positive role in both CHS and epicutaneous immunization, but only under certain conditions (low sensitizing dose and flank route of exposure). These results reconcile disparate findings about LC in the literature.

Materials and Methods

In vivo depletion of LCs

Lang-DTR mice and Lang-EGFP transgenic mice have been described (11) and were crossed to generate F1 Lang-DTR enhanced GFP mice in our
Contact hypersensitivity

All mice were shaved at least 2 days before immunization. Twenty-five μl of 0.3% dinitrofluorobenzene (DNFB) (Sigma-Aldrich) in a mixture of acetone and olive oil (4/1) was painted on the back flank of the mice. On day 5, all of mice were challenged with 5 μl of 0.15% DNFB on both sides of one ear. Ear thickness was measured before and 24 h after challenge, with a spring-loaded micrometer (Mitutoyo).

LC isolation and detection

Epidermal cell suspensions were prepared by ear skin by limited trypsinization and dissociation of epidermal sheets by pipetting in DNase as previously described (18). Cells were then labeled with anti-CD45.2, anti-I-A^d (both BD Pharmingen). DC from lymph nodes were prepared by digestion with collagenase D (Sigma-Aldrich) and EDTA as previously described (19) and cells were stained with anti-CD11c, anti-CD8, anti-I-A^d, and allophycocyanin-coupled Abs to CD3 and NK1.1. All data were collected on a LSRII (BD Biosciences).

T cell adoptive transfer

CD4^+CD8^− Thy1.1^+ OTI T cells were purified by negative depletion using magnetic cell sorting MACS (Miltenyi Biotec) as previously described (20). Cells were collected from lymph nodes and labeled with FITC-coupled Abs to CD4, anti-B220, I-Ab (AF6–120.1), and CD44 (DM-7) (0.0125 μg anti-CD4, anti-B220 and anti-I-A^d per 1 × 10^6 cells; 0.004 μg anti-CD44 per 1 × 10^6 cells) (all from BD Pharmingen). Following staining, cells were subject to depletion using anti-FITC MACs microbeads. Cell purity (>90%) was established by flow cytometry. In some experiments, purified OT-I cells were labeled with CFSE (Molecular Probes) as previously described (21). A total of 2.5 × 10^5 purified CD4^+CD8^− Thy1.1^+ OTI T cells were injected into the tail vein of recipient mice. At various times, single cell suspensions from s.c. lymph nodes and spleen were stained with anti-Thy1.1, anti-CD8 (all from BD Pharmingen) to detect transferred cells.

Epicutaneous immunization and FITC-OVA or tetramethylrhodamine isothiocyanate (TRITC) painting

Mice using for epicutaneous immunization were shaved 2 days before and transferred with purified OTI cells 1 day before immunization. On the day of immunization mice were anesthetized with ketamine and xylazine (100/10 μg/kg body weight) and the flanks and/or ears were shaved for 15 min with water. The hydrated area were painted with 10 μg OVA and 469 μg OVA in 25 μl PBS (Sigma-Aldrich), respectively. These mice were then covered with an occlusive patch (DuoDERM Extra Thin, ConvaTec). The occlusive patch was left in place for 2–4 days. The control mice were painted with PBS.

In FITC-OVA painting experiments, mice were prepared as above mentioned and painted on the ears or flank with 500 μg FITC-OVA. Eight hours later, the skin biopsies were harvested and the fluorescence on the skin sections was detected using confocal microscopy. The green dots were counted in at least 20 images per group. In TRITC painting experiments, a stock solution of 10% TRITC (Molecular Probes) in DMSO was diluted to 1% in a solvent 50/50 (vol/vol) of acetone and dibutylphthalate just before application, and mice were prepared as above mentioned and painted on the limited area of the flanks and ears at the same time. The cervical and inguinal skin draining lymph nodes were harvested and skin non-draining lymph nodes served as a control.

Results

Langerin positive cells promote the contact hypersensitivity response to hapten

To determine the role of LC in the processing and presentation of skin Ags, we used Lang-DTREGFP mice (11), in which LC can be acutely depleted by administration of DT. We depleted LC by i.p. injection of two doses of 1 μg DT at 4 and 1 days before priming, in a standard contact hypersensitivity assay using the hapten DNFB (Fig. 1A). We observed a substantial (95%) reproducible ablation of the CHS response (Fig. 1B). This was surprising, given the previous finding with these mice (11), that LC were dispensable for priming the contact hypersensitivity response to hapten. However, our protocol was slightly different from that used previously. In that study, toxin was administered 3 days before and 1 day after priming (−3/+1) (see Fig. 1A). The protocol in this study administered toxin 4 and 1 days before priming (−4/+1). We did this for two reasons. First, we wished to deplete LC immediately before priming (day −1, as opposed to day −3), and not risk the possibility that re-emerging LC (or LC precursors) could participate in priming. Second, we avoided toxin administration after priming, in case a newly generated LC (or LC precursors) would be exposed to the immunogen and then die, potentially acting as a novel Ag depot via uptake by other DC. To determine whether toxin timing was a relevant variable, we directly compared the two ablation protocols. CHS was diminished when LC were depleted on day −4/−1 (p = 0.0117). In contrast, a slight decrease was observed when LC were depleted on day −3/+1 but this was not statistically significant (p = 0.1479), consistent with the previous report (11). Thus, the precise timing of toxin delivery makes a substantial difference in terms of the CHS response.

The fact that small differences in toxin deliver immediately before priming had a critical effect is surprising given that epidermal LC were reported to be undetectable for up to 14 days after toxin administration in these mice (11). Indeed, we confirmed that epidermal LC were >97% depleted in sheets purified from either the flank or ear up to 14 days after a single injection of toxin in these mice (Fig. 2B and data not shown). Nonetheless, acute toxin administration impairs CHS, while administration of toxin 3 days or more before priming does not result in an impaired CHS response (Fig. 1B and data not shown). These data strongly suggest that epidermal Langerhans cells are not the only Langerin^+ APCs that participate in skin immune responses. We recently discovered a novel population of Langerin^+ DC that reside in the dermis (22). This population is depleted in Lang-DTR mice, but recovers much more rapidly than epidermal LC. In fact, this population is starting to recover by day 3 in the dermis and we found that inhibition of CHS was reversed when DT was administrated on −3 or −7 before immunization (22) (Fig. 1B), suggesting that dermal DC can play an activating role in CHS.
FIGURE 2. Langerin positive cells facilitate Ag presentation to T cells in the skin draining lymph node. A, Experimental scheme for the depletion of LC in an epicutaneous immunization assay. Mice were transferred with 2.2 × 10^6 CFSE-labeled OT-I Thy1.1^+ CD8 T cells 24 h before epicutaneous immunization with OVA protein or peptide (see Materials and Methods for immunization details). Some groups were depleted of LC with DT on days −4/−1 before immunization. The efficacy of LC depletion is shown in B and the result is representative of four experiments. The left panel shows the fraction of GFP^+ cells recovered from epidermal sheets 24 h after toxin treatment. The right panel shows the relative number of GFP^bright^CD8^bright^ (LC) or GFP^dim^CD8^dim^ CD8^+^ cells recovered from skin draining lymph nodes after 24 h. C, Mice were then immunized epicutaneously with either 10 μg OVA protein or 469 μg OVA protein in the absence of an adjuvant. The negative control represents mice that were anesthetized and shaved, but not immunized. The expansion of OT-I T cells was measured 5 days after immunization. Each experiment had three mice/group. Error bars indicate SD. D, shows representative histograms of CFSE dye dilution after gating on Thy1.1^+^ CD8 T cells at day 5. E, Granzyme B production at day 5. F, Groups of mice were depleted LC with DT either on −1 or on −7 before immunization. Mice were then immunized with OVA protein on the flank and OTI cells expansion was measured 5 days after immunization. The data are from two experiments with four mice/group.

Langerin positive cells promote T cell proliferation to epicutaneous Ag under some conditions

DCs can promote adaptive immune responses via multiple mechanisms (23). In addition to processing and presenting Ags to T cells, DC can respond to infection and injury cues, and promote inflammation via recruitment of other cells. Langerin^+^ cells might contribute to CHS priming via any or all of these mechanisms. Because the specific Ags that T cells recognize in a hapten specific CHS response are not defined, we used an epicutaneous protein immunization strategy to ask whether LC are involved in Ag processing and presentation. In this study, mice were transferred with Ag specific OTI CD8 T cells, followed by ear or flank immunization with 469 μg of OVA protein (OVA) or equal moles of OVA^257-264^ (SIINFEKL) peptide (OVAp) with no adjuvant. This protocol results in ~10-fold clonal expansion of OT-I that peaks at day 6, but poor development of effector function such as granzyme B up-regulation (Fig. 2E) and IFNγ production (data not shown). In the case of ear immunization, the elimination of LC had no significant effect on expansion of OT-I T cells (Fig. 2C). This was the case whether peptide or protein were used as the immunogen. Similarly, when peptide Ag was applied on the flank, the absence of LC did not affect the OT-I response. However, LC depletion did affect OT-I expansion when OVA protein was administered via the flank skin (Fig. 2C). Although some proliferation of OT-I T cells could be observed under these conditions (Fig. 2C), the average reduction in expansion was 85% over four individual experiments (data not show). This data suggested that LC can and do play a role in promoting immunity to Ags administered by the skin, although this is dependent on the nature of the Ag (OVAp vs protein) and by the immunization route (flank vs ear).

To determine whether dermal or epidermal Langerin^+^ cells mediate this response, we injected toxin either immediately before (−1) or 1 wk before (−7). As described earlier, 1 wk after toxin delivery Lang-DTR mice have no detectable recovery of epidermal LC. However, 40% of dermal Langerin^+^ cells have recovered by then. OT-I T cell expansion was inhibited when LC were depleted immediately before flank immunization, but not when LC were depleted on day −7 (Fig. 2F) suggesting that dermal Langerin^+^ DC play a role in promoting T cell activation after epicutaneous immunization.

OVA peptide and protein activate OTI T cells with different kinetics

One of the goals of epicutaneous immunization is to have the immunogen break through the stratum corneum barrier and efficiently penetrate into the deeper layers of the skin (24). However, to mimic steady-state conditions, we did not use tape stripping or any other strategies to disrupt this natural barrier. An obvious difference between protein and OVAp is their molecular size, thus it is possible that peptide Ag could more efficiently gain access to the deeper layers of the skin, or soluble peptide might even drain into lymph nodes via lymphatics (25). Thus, it could have a greater chance of being presented by other subsets of DCs. In contrast, protein Ag may stay in the outer layer of the skin, especially in the flank where the keratinized layer is thicker, thus confining Ag presentation to LC. It is known that DCs residing in the dermis migrate to skin draining lymph nodes much more rapidly that LC (11). Thus, we tested this idea by comparing the kinetics of the OT-I response to protein and OVAp. CD69 was up-regulated on transferred OT-I cells from skin draining lymph nodes at 24 h after OVAp immunization and increased at 48 h (Fig. 3A). In contrast, CD69 up-regulation was first observed 48 h after protein immunization (Fig. 3B). CD69 was not up-regulated on OT-I T cells
from non-draining nodes. Corresponding with activation, OT-I expansion reached its peak on day 4 after immunization to OVAp, 2 days earlier than the response to protein (Fig. 3C). The kinetic data do not rule out that LC participate as APCs in the response to peptide epicutaneous immunization (ECI). Rather, they suggest that LC are not essential, because peptide gains access to a more rapidly migrating DC population in addition to LC.

**LC play a more prominent role in flank than in ear**

In addition to a differential role between peptide and protein, we observed that the response to ECI protein was dependent on LC when it was applied to the flank, but not when applied to the ear (Fig. 2, C and D). Nonetheless, the keratinized layer is thicker on the flank than on the ear. Thus, Ags painted on the ear might more easily penetrate into the skin, cross the basement membrane, and have an increased propensity to be transported by other DCs, such as dermal DC. Indeed, FITC-OVA applied to the skin was detectable in the dermis of the ear but not the flank 8 h later (Fig. 3D), although this does not address whether Ag was processed and presented. To address this, we characterized the kinetics of the OT-I response as above. CD69 was dramatically up-regulated on transferred OT-I cells from ear draining lymph nodes 24 h after OVA protein immunization (Fig. 3E). In contrast, CD69 up-regulation was first observed only 48 h after protein immunization on the flank (Fig. 3, A and E). Again, CD69 was not up-regulated on OT-I cells from non-draining nodes (data not shown). Because dermal APC migrate more rapidly than epidermal, this suggests the involvement of a dermal APC in the ear. As above, this suggests that LC are not essential for epicutaneous immunization on the ear because the Ag gains access to a more rapidly migrating DC population in addition to LC.

To investigate whether ear skin has a more rapidly migrating DC population in addition to LC, Lang-DTREGFP mice were painted with the fluorescent dye TRITC, both on the ears and on the flank. At various time points later, the ratio of LC to other subsets of DC among total TRITC plus DC was determined. At each time point, the ratio of LC to “other DC” was significantly higher among TRITC+ cells in the flank compared with the ear (Fig. 4B). As mentioned earlier, LC migrate more slowly than dermal DC, so the fraction of TRITC+ cells that are LC increased over time (Fig. 4B). However, even at these later time points, DC trafficking from the ear contained a lower representation of LC than from the flank. We also noticed that the epidermis of ear contains a notable population of hematopoetic cells recruited to the

**FIGURE 3.** Ag recognition occurs more quickly in LC independent responses. Mice were transferred with 2.5–10 × 10⁶ OTI cells and immunized with either OVAp or OVA protein on the flank (A–C) or flank and ear (E). Cells from the skin draining lymph nodes and non-draining nodes were harvested separately and CD69 expression on the transferred cells was compared 24 and 48 h after immunization (A, B, and E). The histograms in A and B are representative of at least five experiments performed, and the histograms in E are representative of two experiments performed (n = 3 per group). The expansion of OT-I T cells over time to protein and peptide immunization on the flank is shown in C and the result is representative of three experiments. D, Five hundred μg of FITC-OVA was applied to hydrated skin at the ear or shaved flank. 8 h later, skin biopsies were harvested for analysis by immunofluorescence. The number of FITC+ events in the dermis per image was quantitated. Error bars indicate SD of in 20 images from three animals per group.

**FIGURE 4.** Ear skin has a greater proportion of Langerin negative APC. Mice were painted on the ear or the flank with TRITC. At 24, 48, 72, or 96 h later, the cervical lymph node or the inguinal lymph node draining the TRITC site were harvested. A, Gated I-A b high TRITC+ cells 48 h after TRITC painting. The histogram shows the proportions of EGFP+/− population in the I-A b high TRITC+ gate. B, Ratio of EGFP+ (LC) to EGFP− (non LC) cells after gating on TRITC+ I-A b high cells. Analysis was performed on two mice/group at each time point, in two independent experiments. C, Total epidermal cells were recovered 24 or 48 h after epicutaneous immunization with OVA protein alone on the flank or ear. The bar graphs show an increased proportion of hematopoietic cells (CD45+ non LC) cells after gating on TRITC+ in the ear immunization site, but not flank. Error bars indicate the SD. D, A flow cytometric graph shows expression of I-A b and Gr1 on CD45+ cells from ear skin 48 h after immunization.
site after epicutaneous immunization (Fig. 4C). This population includes I-A^B^+ GR-1^- cells, which could have Ag presenting function (Fig. 4D). Thus, it would appear that Ags applied through ear skin potentially have access to more diverse DC, and may explain why LC ablation had no effect on protein ECI at the ear.

Discussion

A limited immune-promoting role for Langerin^+ cells

There is significant interest in developing epicutaneous vaccination because of the potential for self-administration, ease of delivery and stockpiling, and lack of systemic side effects (26, 27). This strategy, delivery of a protein Ag after skin hydration or mild abrasion, has been shown to be effective for multiples Ags and provides protective immunity against virus infection (28, 29). Its efficacy has been attributed to the potency of Langerhans cells in stimulating T cell responses. Yet, to our knowledge, this study is the first to address whether LC play a role epicutaneous immunization strategies.

We were able to demonstrate a role for Langerin^+ cells in ECI, as depletion resulted in an 85% inhibition of OT-I clonal expansion when OVA protein was delivered to the shaved flank and covered with a bandage. However, Langerin^+ cells were not required for ECI in all circumstances. In particular, peptide-induced ECI occurred normally in the absence of LC. This is likely due to the involvement of additional APC when peptide is available for loading at the cell surface. Indeed, we observed T cell activation at 24 h after peptide immunization, which is generally inconsistent with LC delivery, as they are thought to migrate to lymph nodes with substantially delayed kinetics compared with dermal DC (11). It is possible that some peptide may even drain to the skin draining lymph nodes via lymphatics, although this seems unlikely to us because in that case the response was as early as 30 min after Ag injection (25). The kinetics of T cell activation after protein ECI were more consistent with a role for LC, because T cell activation was not observed until 48 h.

We also showed that protein ECI, when applied to the ear, was independent of LC. However, using cell tracking with TRITC, we showed that LC represent a smaller fraction of the migrating APC in the ear compared with flank, and this may explain why ear ECI is not inhibited by LC elimination. Possible candidate skin APC that could migrate to skin draining lymph nodes and present Ag are dermal DCs and inflammatory monocytes, which can be recruited to sites of inflammation and injury and differentiate into DC (30).

Our findings are in agreement with the recent study of Bennett et al. (31) who used a similar Langerin-DTR model to show that topically applied FITC is inefficiently transported to the skin draining lymph nodes after depletion of Langerin^+ cells. In contrast, LC were shown not to play a role in gene gun immunization on the abdominal skin (32). However, in gene gun immunization, the DNA is likely to penetrate deeper in the tissue than with epicutaneous immunization, and in this manner may target distinct APC.

Overall, our findings suggest that LC are not playing a unique and/or critical role in epicutaneous immunity, but rather their contribution to epidermal immune responses depends on the specifics of Ag delivery and lymph drainage.

Reconciling conflicting data on the role of LC in CHS

Our findings also shed light on the recent controversy regarding the role of LC in the contact hypersensitive response. We showed that CHS could be blocked by LC depletion in Lang-DTR mice. This contrasts with the original report on these mice. However, the difference likely is due to slight differences in the hapten dose and timing of toxin delivery. We compared the protocol previously used (at days -3 and +1 relative to the hapten priming step), to one where toxin was delivered 24 h before the hapten priming (at days -4 and -1). The CHS block was observed only in the latter. Interestingly, in both situations epidermal LC were completely depleted (and remained so for at least 14 days). This raises the possibility that CD8^+ Langerin^+DC are required for CHS. Although possible, we consider this unlikely. For one, the depletion of this population with toxin is not complete, presumably owing to the lower level of DTR expressed. Fifteen percent of CD8^+ GFP cells were present in s.c. lymph nodes, even after acute high dose toxin administration (Fig. 2B and data not shown). Second, this population is not present in the skin, and traffics to s.c. lymph nodes directly via the blood (33).

Another explanation that we consider more likely is that there is another Langerin^+ cell in the skin besides the epidermal LC and that such a cell could be replenished in the skin after the -3/+1 protocol but not the -4/-1 protocol. Indeed, we recently published evidence that a Langerin positive cell resides in the dermis and migrates to skin draining lymph nodes in the steady state (22). After depletion in Lang-DTR mice, it rapidly reconstitutes the dermis, in contrast to epidermal LC, which are not reconstituted for weeks. We showed that this dermal Langerin^+ cell can contribute to CHS (22), and we show in this study that it can contribute to the T cell response to epicutaneous OVA. We also showed that in a separate model of LC depletion, the hLang-DTA mice (13), that dermal Langerin^+ cells are still present. Presumably this is due to species specific differences between the human and murine Langerin gene expression. This finding can explain why CHS is not impaired in hLang-DTA mice.

Nonetheless, one aspect of these findings remain difficult to reconcile with the results of Kaplan et al. (13), who showed that CHS is enhanced in hLang-DTA mice. It is worth emphasizing that depletion of epidermal LC in our studies was profound, and yet we did not ever observe the enhancement of either CHS or ECI responses. However, the absence of LC from birth in the hLang-DTA mice might establish a novel compensatory mechanism to facilitate epidermal immune responses that does not occur in normal mice. Thus acute elimination of LC might yield different results than chronic elimination. Further study is needed to test this possibility.

In summary, acute depletion of Langerin positive DCs using Lang-DTR mice was used to show that Langerin positive cells do promote immune responses to skin Ags under limited conditions. We provide reconciliation of disparate data from two different acute depletion models by showing that the timing of toxin administration is critical to observing reduced CHS. Furthermore, our findings also reveal heterogeneity among Langerin positive cells, and suggest that this heterogeneity may be functionally relevant to skin immune responses.

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Disclosures

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